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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Recently breast cancer resistance protein (BCRP) has been found to be a frequent cause of MDR by causing increased efflux of a wide variety of cytotoxic drugs. Although it has been shown that transfection of BCRP into breast cancer cell line MCF7 caused drug resistance, it has also been found that the drug resistance level of these cells were much lower than that of the drug-selected cells. Thus, there must be other drug resistant mechanisms in the drug selected MCF7/AdrVp cells. This study is designed to test this concept.

Specifically, we plan to achieve the following objectives using proteomics technology: (a) to compare protein profiles between MCF7 and MCF7/AdrVp cells using two-dimensional gel analysis, (b) to identify the proteins of different levels between the two cell lines using MALDI-TOF mass spectrometry analysis, (c) to confirm the different level of the identified proteins using western blot, and (d) to test the role of these proteins in mediating MDR using MTT assay.

The information and probes obtained from this study will help us understand the molecular mechanism of drug resistance in breast cancer cells. This work may also help us discover new therapeutics for treating drug resistant breast tumors.

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INTRODUCTION

The use of anticancer agents in appropriate combinations has led to major improvements in the treatment of malignant tumors. Previously fatal diseases, such as Hodgkin's disease, are now curable while others, such as breast cancer, can undergo remission. Resistance to chemotherapy frequently occurs in breast cancers and is a major obstacle to successful breast cancer treatment. Studies with tumor cell lines such as MCF7 have revealed that multidrug resistance (MDR) can develop and thus cause chemotherapy failure. Advances in elucidating the molecular basis of the MDR phenotype indicate that expression of P-glycoprotein (Pgp) and multidrug resistance protein 1 (MRP1) is a frequent cause of MDR in human breast cancers (Ambudkar et al., 1999). Recently, another membrane protein, breast cancer resistance protein (BCRP), has also been found to be a frequent cause of MDR (Doyle et al., 1998)-(Miyake et al., 1999). Pgp, MRP1 and BCRP all belong to the ATP-binding cassette transporter superfamily (Dean et al., 2001). Cancer cells over-expressing Pgp, MRP1, or BCRP have an increased ability to efflux a wide variety of cytotoxic drugs and, therefore, can survive chemotherapy (Gottesman et al., 2002).

BODY

This progress report is for a concept award. In the original application, we proposed to accomplish the following objectives: (a) to compare protein profiles between MCF7 and MCF7/AdrVp cells using two-dimensional gel analysis, (b) to identify the proteins of different levels between the two cell lines using MALDI-TOF mass spectrometry analysis, (c) to confirm the different level of the identified proteins using western blot, and (d) to test the role of these proteins in mediating MDR using MTT assay.

We have accomplished most of our studies as planned. Firstly, a regular SDS-PAGE was performed and a protein of 275 kDa was found over-expressed (see Figure 1 in the poster appended). This protein was later identified to be fatty acid synthase by MALDI-TOF mass spectrometry (see Table 1 in the poster appended). A two dimensional gel electrophoresis was then conducted on the cell lysates prepared from the parental drug sensitive MCF7 cells and the drug-selected MCF7/AdrVp3000 cells and 17 protein spots were found to be differentially expressed between the two cell lines (see Figure 2 in the poster appended) and were identified by MALDI-TOF mass spectrometry (see Table 1 in the poster appended). We then confirmed the expression level of some of these proteins using western blot and real time RT PCR (see Figures 4 and 5 in the poster appended). We are currently in the process of testing whether the altered expression of these proteins plays any role in drug resistance in breast cancer cells.

KEY RESEARCH ACCOMPLISHMENTS

1. Seventeen proteins were identified which have differential expression levels between the drug sensitive parental MCF7 and the drug resistant MCF7/AdrVp3000 cells.
2. The differential expression levels of some of these proteins were confirmed by western blot and/or real time RT PCR.

REPORTABLE OUTCOMES

1. Liu, Y.; Liu, H.;, Zhang, J.-T. Proteomic analysis of drug resistant breast cancer cell line MCF7/AdrVp3000 (2004). Proceedings of American Association of Cancer Research 45 (http://aacr04.agora.com/planner/displayabstract.asp?presentationid=3465).

CONCLUSIONS

In conclusion, at least 17 proteins have altered expression level in the drug selected MCF7/AdrVp3000 cells compared with the parental drug sensitive MCF7 cells. This observation suggests that other mechanisms are likely also responsible for drug resistance of MCF7/AdrVp3000 cells in addition to the known the increased drug efflux due to elevated expression of BCRP. We are currently testing these possibilities.

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Proteomic Analysis of Drug Resistant Breast Cancer Cell Line MCF7/AdrVp3000

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ABSTRACT

MCF-7/AdrVp3000, a drug resistant human breast cancer cell line derived from parental MCF-7 cells by stepwise selection with adriamycin in the presence of P-glycoprotein inhibitor verapamil, has been shown to over-express an ABC-transporter ABCG2 which was thought to cause the observed drug resistance phenotype in MCF7/AdrVp3000 cells. However, MCF7 cells with similar ABCG2 level by enforced ectopic expression did not produce similar level of drug resistance, suggesting that other mechanism of resistance may have been selected in MCF7/AdrVp3000 cells. In this study, we used proteomic approach to compare the global protein profile between MCF-7 and MCF-7/AdrVp3000 cells. Following two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, 17 proteins with differential levels between the two cell lines were identified. Twelve proteins including cytokaratin 18, cytokaratin 19, 14-3-3σ, ATPase β subunit, protein disulfide isomerase (PDI), HSP27, cathepsin D, triosephosphate isomerase 1, peroxiredoxin 6, electron transfer flavoprotein and fatty-acid synthase were found over-expressed in MCF-7/AdrVp3000 cells. Other proteins including non-metastatic cells 1 protein, peroxiredoxin 2, nucleophosmin 1 and inorganic pyrophosphatase were found decreased in MCF-7/AdrVp3000 cells. The different levels of these proteins between the two cell lines were confirmed by western blot and/or real time RT-PCR. The differential expression of these proteins may also be responsible for the drug resistance in MCF7/Adr/Vp3000 cells selected by adriamycin.

INTRODUCTION

A major obstacle in the efficient chemotherapy of human cancer is the intrinsic or acquired multidrug resistance (MDR) to cytostatic and cytotoxic drugs. To study the mechanisms of drug resistance, many drug resistant cell lines have been developed *in vitro* by selecting with various agents. A drug resistant breast cancer cell line, MCF-7/AdrVp3000, was isolated from parental MCF-7 cells by stepwise selection with adriamycin in the presence of P-glycoprotein inhibitor verapamil. MCF-7/AdrVp3000 displays an ATP-dependent reduction in the intracellular accumulation of anthracycline anticancer drugs in the absence of over-expression of known multidrug resistance transporters such as P-glycoprotein or the multidrug resistance-associated protein. A half ABC transporter, ABCG2, was shown to be over-expressed in this cell line. MCF7 cells transfected with ABCG2 cDNA showed similar profile but with a much reduced level of drug resistance when compared with that of MCF-7/AdrVp3000 cells. In addition, two non-drug resistance proteins, H19 gene and NCA-90 (nonspecific cross-reacting antigen), were also identified to be highly expressed in MCF-7/AdrVp cells, a derivative cell line from the early step of selection. In an attempt to investigate whether other mechanisms may have been selected by adriamycin in MCF-7/AdrVp3000 cells but absent in the parental MCF-7 cells, we applied the proteomic approach which combines two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry to compared the global protein profile and to identify the proteins with differential expression between MCF-7 and MCF-7/AdrVp3000 cells .

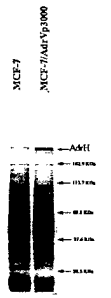

Figure 1. SDS-PAGE analysis of MCF-7 and MCF-7/AdrVp3000 cell extracts. MCF-7 and MCF-7/AdrVp3000 cells grown in IMEM were extracted in TNN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 20 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 2 mM PMSF and 0.1% SDS) by sonication. After centrifugation at 10,000g for 10 min, 20 μg proteins from the supernatant were separated by a precast 4~15% SDS-PAGE and stained by commassie blue. A high molecular weight protein (shown as AdrH) was found to have an elevated level in MCF-7/AdrVp3000 cells which was identified as fatty acid synthase by MALDI-TOF mass spectrometry (Accession No. G01880).

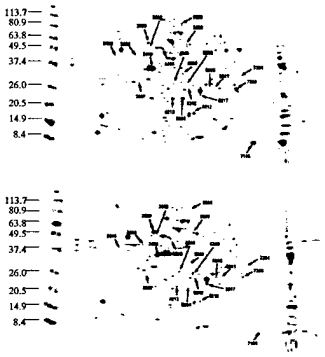

Figure 2. Two-dimensional gel electrophoresis profile of MCF-7 and MCF-7/AdrVp3000 cells. 120 μg proteins of MCF-7 (panel A) and MCF-7/AdrVp3000 (panel B) extracts were first separated by IEF (pH 3-10) followed by SDS-PAGE (10-20% gradient gel) and stained with commassie blue. The protein profile were analyzed using a PDQuest software (Bio-Rad). The numbered spots were identified by MALDI-TOF mass spectrametry analysis.

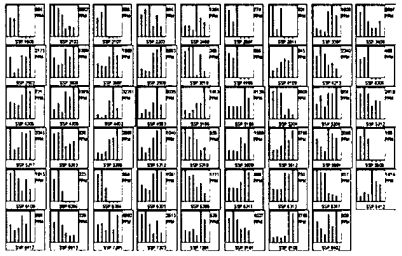

Figure 3. Quantitative levels of protein spots with differential expression between MCF-7 and MCF-7/AdrVp3000 cells. Individual bars represent the protein quantity value of each gel of three different gels. The left three bars correspond to MCF-7 and the right three bars correspond to MCF-7/AdrVp3000 cells. SSP numbers represent the spot number of each protein. The value in y-axis is depicted as parts per million (ppm) determined using PDQuest gel analysis software.

Identified proteins with different levels between MCF-7 and MCF-7/AdrVp3000 cells

SSP No.	Expression level	Protein Name	Accession No.	MW (kDa)	pI	Coverage (%)[a]	Z Score[b]
2408	Up in MCF-7/AdrVp3000	Keratin 19	NP_002267	44 09	5 0	24	2 18
3307	Up in MCF-7/AdrVp3000	14-3-3 σ	NP_006133	27 87	4 7	50	2 31
3506	Up in MCF-7/AdrVp3000	Keratin 18	NP_000215	48 04	5 3	23	2 35
3509	Up in MCF-7/AdrVp3000	Protein disulfide-isomerase precursor (PDI)	P07237	57.5	4 8	46	2 38
4503	Up in MCF-7/AdrVp3000	ATP synthase β subunit	P06576	56 54	5 3	48	2 29
5217	Up in MCF-7/AdrVp3000	Heat shock 27kD protein	NP_001531	22 82	6 0	49	2 36
5312	Up in MCF-7/AdrVp3000	Heat shock 27kD protein	NP_001531	22 82	6 0	33	1 33
5308	Up in MCF-7/AdrVp3000	Cathepsin D, Chain B	1LYW_B	26 46	5 3	32	1 74
4309	Up in MCF-7/AdrVp3000	Cathepsin D, Chain B	1LYW_B	26 46	5 3	24	1 47
5509	Up in MCF-7/AdrVp3000	Protein disulfide-isomerase precursor (PDI)	P07237	57.5	4 8	25	2 31
6305	Up in MCF-7/AdrVp3000	Triosephosphate isomerase 1	NP_000356	26 84	6 4	57	2 35
6311	Up in MCF-7/AdrVp3000	Peroxiredoxin 6	NP_004896	25 13	6 0	37	1 81
7304	Up in MCF-7/AdrVp3000	Human Electron Transfer Flavoprotein	1EFV_A	33.42	7 0	44	1 65
7105	Up in MCF-7/AdrVp3000	Keratin 19	NP_002267	44 09	5 0	34	1 85
3408	Up in MCF-7	Nucleophosmin 1/B23	NP_002511	32 79	4 6	39	1 36
3510	Up in MCF-7	Tubulin, β	138369	49.38	4 7	22	1 03
4213	Up in MCF-7	Thioredoxin Peroxidase B	1QMV_A	21 68	5 7	38	2 2
5204	Up in MCF-7	Thioredoxin Peroxidase B	1QMV_A	21 68	5 7	34	2 27
5212	Up in MCF-7	non-metastatic cells 1 protein (nm23)	NP_000260	17 3	5 8	57	2 34
5303	Up in MCF-7	inorganic pyrophosphatase	XP_045578	33.13	5 5	32	1 73
7303	Up in MCF-7	triosephosphate isomerase 1	NP_000356	26 84	6 4	71	2 39
5608	Only in MCF-7	Heat shock 90 KD protein	NP_005339	85 04	4 9	17	1 1
AdrH[c]	Up in MCF-7/AdrVp3000	Fatty acid synthase	G01880	275 70	6 0	5	1 69

Protein spots from triplicate gels were excised, alkylated with 55 mM iodoacetamide and digested with trypsin (6 ng/ μl) overnight at 37°C followed by MALDI-TOF mass analysis. The measured peptide mass profiles were then compared with the theoretical peptide masses using ProFound search engine and NCBI database for protein identity.

[a] Coverage is defined as the ratio of the portion of protein sequence covered by matched peptides to the full length sequence of the protein.

[b] Z score is an indicator of the quality of the search result which is estimated when the search result is compared against an estimated random match population. Z score is the distance to the population mean in unit of standard deviation. It also corresponds to the percentile of the search in the random match population.

[c] AdrH is the protein found highly expression in MCF-7/AdrVp3000 cells from SDS-PAGE.

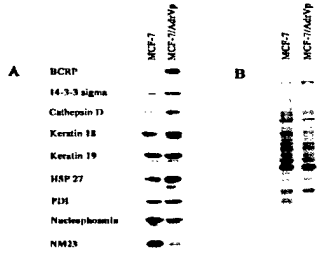

Figure 4. Western blot analysis. Same amount of lysates from MCF7 and MCF7/AdrVp3000 cells were separated by SDS-PAGE followed by western blot analysis (A) for ABCG2 (BCRP), 14-3-3 σ, cathepsin D, keratin 18, keratin 19, protein disulfide-isomerase precursor (PDI), heat shock protein (HSP27), nucleophosmin 1, and NM23 or for staining with commassie blue (B).

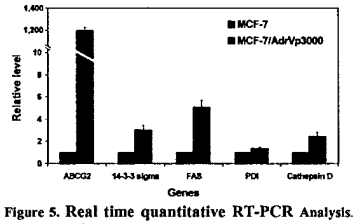

Figure 5. Real time quantitative RT-PCR Analysis. Total RNAs isolated from MCF-7 and MDF-7/AdrVp3000 cell were reverse transcribed using AMV reverse transcriptase and Oligo$(dT)_{12-18}$ primers. Real time quantitative PCR was carried out using gene specific primers. Relative mRNA levels were measured using SYBR Green and calculated in the fold change ($2^{\Delta Ct}$) relative to MCF-7 cells after normalized by the internal control, GAPDH

SUMMARY

- Both one-dimensional and two-dimensional gel electrophoresis showed different protein expression profile between MCF-7 and MCF-7/AdrVp3000 cell lines.
- Fifty-three differentially expressed protein spots were exercised from 2D gels and analyzed by peptide mass fingerprinting. Of these proteins, 17 were identified using ProFound search engine by comparing with the theoretical peptide masses from NCBI database.
- Of the 17 identified proteins, 9 were confirmed by western blot and/or real time quantitative RT-PCR.
- These proteins with differential expression between the two cell lines may be responsible in part for the drug resistance selected in MCF7/AdrVp3000 cells.